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IMPACT OF CANDIDATE GENES ON OBESITY AND TYPE 2 DIABETES

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ABSTRACT

Obesity and type 2 diabetes (T2D) have strong genetic components. Identification of susceptibility genes in both diseases will give better knowledge of their pathomechanisms and future therapies. In this thesis, the candidate gene approach was used to find genetic variations associated with obesity and T2D in a Swedish population, including controls with normal glucose tolerance (NGT), subjects with impaired glucose tolerance (IGT), patients with T2D and obese subjects. The candidate genes were selected based on previous studies and on their role in biological pathways relevant for determination of body composition and glucose homeostasis.

Receptor protein tyrosine phosphatase sigma (RPTPσ) **(paper I)** has a function in cellular receptor signaling and is highly expressed in insulin target tissues. We have previously shown that *RPTPσ* is over-expressed in pancreatic islets and liver of spontaneously diabetic Goto–Kakizaki (GK) rats. Single nucleotide polymorphisms (SNPs) in the *RPTPσ* gene were genotyped in NGT subjects, IGT subjects, and T2D patients. Three polymorphisms conferred susceptibility to T2D. SNP rs1143699 was associated with an increased risk of T2D in male patients carrying the C/C genotype. SNPs rs4807015 and rs1978237 were associated with an increased risk of T2D in both male and female patients.

Zn-α_2-glycoprotein 1 (AZGP1) **(paper II)** is a novel adipokine that may be involved in the regulation of body weight. Through microarray gene expression we found that the *AZGP1* gene was down-regulated 3.9-fold in subcutaneous (S.C.) abdominal fat of NGT obese subjects compared to lean. We also showed that *AZGP1* is significantly decreased in S.C. abdominal and omental fat but not in S.C. thigh fat. Genotyping of *AZGP1* polymorphisms in NGT lean subjects, NGT obese subjects, IGT obese subjects and T2D patients revealed that SNP rs2525554 is associated with obesity. Association with the T-allele was evident for BMI, waist circumference, waist-hip ratio and 2h glucose. Decreased *AZGP1* expression in obese subjects was found to correlate with their higher frequency of risk allele T in rs2525554.

Alpha 2-adrenergic receptors are involved in insulin secretion and lipolysis. We evaluated association for the adrenergic receptor alpha 2A (*ADRA2A*) gene **(paper III)** with obesity and/or T2D in our cohort. Data indicate that two SNPs, rs553668 and rs521674, are associated with disease. rs553668 in men is linked to obesity and rs521674 in women to obesity and possibly T2D.

Adenylate cyclase 3 (AC3) **(paper IV)** is expressed in pancreatic islets, brain, heart, kidney, liver, lung and skeletal muscle. A previous study from our laboratory demonstrated that the *AC3* mRNA was overexpressed in the pancreatic islets of the GK rat. In our association study for the *AC3* gene it was found that SNPs rs2033655 and rs1968482 are strongly associated with obesity in NGT subjects and T2D patients. A diplotype analysis with the associated polymorphisms predicted a significant association with BMI in obese subjects.

The results from the four candidate gene association studies have generated knowledge of their role in obesity and T2D development. RPTPσ seems to be involved in T2D whereas AZGP1, ADRA2A and AC3 are most likely linked to obesity. Two studies also revealed gender specific associations. The associated variants need to be investigated further regarding function, gene-gene and gene-environment interactions.

LIST OF PUBLICATIONS

I. **Långberg EC**, Gu HF, Nordman S, Efendic S, Ostenson CG. Genetic variation in receptor protein tyrosine phosphatase σ is associated with type 2 diabetes in Swedish Caucasians. *European Journal of Endocrinology (2007)* ***157*** *(4) 459-464*

II. **Långberg EC**, Lagarde D, Gu HF, Essioux L, Duchateau-Nguyen G, Thorell A, Gardes C, Clerc RG, Ostenson CG. Genetic and functional analyses of Zn-α2-glycoprotein in obesity. *Submitted manuscript*

III. **Långberg EC**, Efendic S, Ostenson CG, Gu HF. Genetic impact of adrenergic receptor alpha 2A on obesity and type 2 diabetes. *Manuscript*

IV. Nordman S, Abulaiti A, Hilding A, **Långberg EC,** Humphreys K, Ostenson CG, Efendic S, Gu HF. Genetic variation of the adenylyl cyclase 3 (AC3) locus and its influence to type 2 diabetes and obesity susceptibility in Swedish men. *International Journal of Obesity (London) (2008)* ***32*** *(3) 407-412*

Publication by the same author:

MacDonald MJ, Longacre MJ, **Långberg EC**, Tibell A, Kendrick MA, Fukao T, Ostenson CG. Decreased levels of metabolic enzymes in pancreatic islets of patients with type 2 diabetes. *Diabetologia (2009)* ***52*** *(6) 1087-1091*

CONTENTS

LIST OF ABBREVIATIONS

AC3	Adenylate cyclase 3
ADHD	Attention deficit hyperactivity disorder
ADRA2A	Adrenergic receptor alpha 2A
ANOVA	Analysis of variance
APS	Adenosine phosphor-sulfate
ATP	Adenosine tri-phosphate
AZGP1	Zinc α_2 glycoprotein 1
BMI	Body mass index
BP	Blood pressure
bp	Base pairs
cAMP	Cyclic adenosine mono-phosphate
CCD	Charge coupled device
CDC	Center for disease control
CI	Confidence interval
CNV	Copy number variant
DASH	Dynamic allele-specific hybridization
DEXA	Dual energy X-ray absorptiometry
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
FAM	Carboxyfluorescein
FDR	False discovery rate
FTO	Fat mass and obesity associated
GIP	Gastric inhibitory polypeptide
GK	Goto-Kakizaki
GLP-1	Glucagon-like peptide 1
GWAS	Genome-wide association studies
HLA-DQB1	Major histocompatibility complex, class II, DQ beta 1
HOMA	Homeostasis model of assessment
HWE	Hardy-Weinberg equilibrium
IDE	Insulin degrading enzyme
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IVT	In vitro transcription
Kb	Kilobase
KCNJ11	Potassium inwardly rectifying channel, subfamily J, member 11
LAR	Leukocyte antigen related
LD	Linkage disequilibrium
LMF	Lipid mobilizing factor
MAF	Minor allele frequency
MODY	Maturity onset diabetes of the young
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
NaOH	Sodium hydroxide
NCBI	National center for biotechnology information

NEGR1	Neural growth regulator 1
NGT	Normal glucose tolerance
NPY	Neuropeptide Y
OGTT	Oral glucose tolerance test
OR	Odds ratio
PCA	Principal component analysis
PCR	Polymerase chain reaction
PPi	Pyrophosphate
PPARG	Peroxisome proliferator-activated receptor gamma
RMA	Rate monotonic analysis
RPTPσ	Receptor protein tyrosine phosphatase sigma
rRNA	Ribosomal ribonucleic acid
SC	Subcutaneous
SD	Standard deviation
SDPP	Stockholm diabetes prevention program
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
T2D	Type 2 diabetes
TAMRA	Tetramethylrhodamine
TCF7L2	Transcription factor 7-like 2
TET	Tetrachlorofluorescein
TG	Triglyceride
TNF-α	Tumor necrosis factor alpha
UCP	Uncoupling protein
UTR	Untranslated region
VLDL	Very low density lipoprotein
VO_2max	Maximal oxygen uptake
WC	Waist circumference
WHO	World health organization
WHR	Waist-hip ratio
QTL	Quantitative trait loci

1 BACKGROUND

1.1 TYPE 2 DIABETES

The worldwide prevalence of diabetes is predicted to increase from 2.8% in 2000 to 4.4% in 2030 with over 350 million people affected [1]. There are two common forms of diabetes, type 1 diabetes (T1D) where the production of insulin is lacking and type 2 diabetes (T2D) caused by β-cell dysfunction and insulin resistance. Both forms leads to hyperglycemia and when poorly treated to increased urine production, compensatory thirst and excessive fluid intake, blurred vision, lethargy and changes in energy metabolism. T2D may be provoked by pregnancy, medications and organic pollutants[2]. Approximately 85-90% of those affected by diabetes have T2D that is a late-onset disease becoming more prevalent due to an aging population and an increase in obesity. It is alarming that obese children and young adults are developing T2D [3]. About 80% of T2D patients are associated with obesity and physical inactivity [4]. Our research group has previously shown that other factors such as tobacco use [5], psychosocial- [6] and work-stress [7] are associated with increased risk of T2D. Another form of diabetes is maturity onset diabetes of the young (MODY). It is characterized by young-age onset, autosomal dominant inheritance and lack of association with obesity. To date, eight genes have been described for MODY.

There are two conditions which usually precede T2D. They are therefore often called pre-diabetes. In impaired glucose tolerance (IGT) an oral glucose load renders plasma glucose levels between normal glucose tolerance and diabetes, and in impaired fasting glucose (IFG), the fasting plasma glucose is in between normal and diabetic values.

Glucose homeostasis is strongly accompanied by the interplay between insulin secretion and insulin actions. During fasting, glucose is mainly produced by the liver and about 50% of it is utilized by the brain. The rest is taken up by other tissues. A normal liver can increase glucose production ≥ 4-fold. In this state plasma insulin is low not to restrain glucose production by the liver. A drop in glucose level also results in the release of glucagon from pancreatic α-cells, which stimulates the conversion of glycogen to glucose. After a meal, insulin levels are increased and glucose production is decreased. The normal pancreatic β-cell can adapt to changes in insulin action i.e. a decrease in insulin sensitivity, insulin resistance, is followed by upregulation of insulin secretion and the other way around [8-10]. When the adaptation is insufficient, individuals will develop pre-diabetes or T2D [11]. Thus, β-cell dysfunction is critical for the pathogenesis of T2D [12]. Insulin resistance occurs when the effects of insulin are abnormal for both glucose disposal in skeletal muscle and/or endogenous glucose production in the liver [11 13]. For a schematic overview of the above mentioned mechanisms, see Figure 1.

T2D complications can be divided into macro- and microvascular. The macrovascular complications occur in larger blood vessels and can lead to cardiovascular disorders including stroke, myocardial infarction and peripheral vascular disease. These complications can happen even with small increases in blood glucose levels. Micro-vascular complications occur in small blood vessels in the eyes, kidneys and peripheral nerves. They are a reflection of the duration and severity of hyperglycemia. The main therapy goal for diabetes is to prevent complications. Besides treatment of hyper-

glycemia patients are usually given anti-hypertensive and anti-hyperlipidemic drugs. Exercise and diet are important for the treatment of T2D and allow simpler regimens to be used to control blood glucose levels [14].

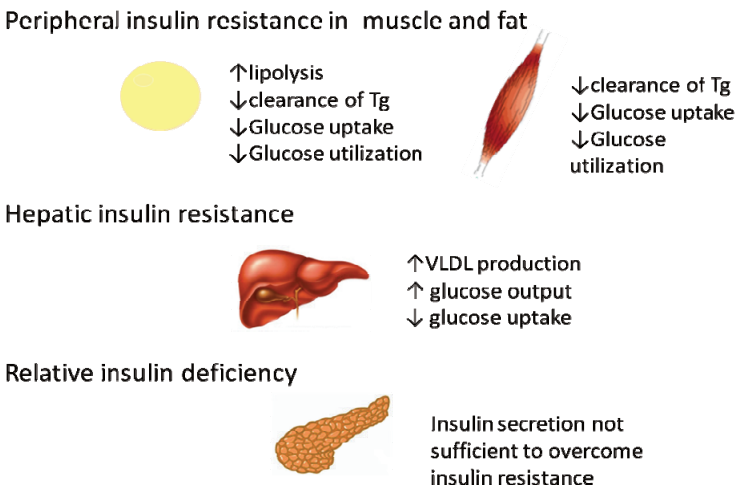

Figure 1. T2D major metabolic defects: increased level of blood glucose is a consequence of impaired insulin action and/or insulin secretion. Insulin resistance is evident when the effects of insulin are abnormal for glucose uptake in skeletal muscle and for glucose production in the liver. TG: triglyceride, VLDL: very low density lipoprotein .

T2D has a strong genetic component which may account for differences in prevalence between ethnic groups, higher concordance rate among monozygotic than dizygotic twins and a sibling risk ratio of about 3.5 [15]. The lifetime risk of T2D is 7% in the general population [16]. A positive family history of T2D confers an increased risk for disease [17] with a 40% risk to develop T2D in first degree relatives. The risk increases to 70% if both parents have diabetes [16].

1.2 OBESITY

Overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health. A common measure of obesity is body mass index (BMI). A person with a BMI of ≥ 30 is generally considered obese. A person with a BMI ≥ 25 is considered overweight. Overweight and obesity are major risk factors for a number of chronic diseases, including diabetes, cardiovascular diseases and cancer [18]. Obesity also contributes to shorter lifespan, depression and decreased quality of life [19-20]. Overweight and obesity are now dramatically on the rise in low- and middle-income countries. The fundamental cause of obesity and overweight is a lack of energy balance between calories consumed and expended. Increases in overweight and obesity are attributable to a global shift in diet towards increased intake of energy-dense foods and a trend towards decreased physical activity.

Global projections estimate 1.12 billion individuals to be obese by the year 2030 [21]. The rapid growth of obesity have occurred in both adults and children [22]. Obesity is a major contributor to morbidity and mortality across the world, surpassing drinking and smoking in its negative effects on health. This will have negative effects on life expectancies of generations born after the rise of the obesity epidemic [23]. The obesity

epidemic during the last decades can not only be explained by our environment. Our genetic background also contributes to this problem [24].

The evidence for a genetic component in obesity is strong [25-26]. The evidence include differences in prevalence between ethnic groups [27-28], higher fat concordance in monozygotic compared to dizygotic twins [29-30] and 30-70% BMI heritability between individuals [31-34]. There are several theories explaining the genetics of obesity but there is no current consensus in the area as a consequence of the complex nature of obesity susceptibility [35]. Common obesity is polygenic with no simple Mendelian inheritance pattern [35]. The theories available overlap to a great extent, but differ in their views of key tissue involved. First, as mentioned previously, obesity has been viewed mainly as a disease of energy balance due to an excess energy intake or a decreased energy expenditure. Second, obesity is also seen as a disorder of the adipocyte as it has a mechanism of fat storage and mobilization. Adipose tissue has been recognized as having an independent endocrine role that can result in an inflammatory response with increased risk of T2D and cardiovascular disease, leading to increased morbidity and early mortality [36]. Adipose tissue affects energy homeostasis and cardiovascular health by releasing adipokines that regulate energy expenditure, food intake, insulin sensitivity and inflammation [36]. Third, a new view of obesity as a neurobehavioral disorder has emerged with the control of appetite and food intake involved in obesity pathogenesis [35 37].

Obesity is strongly associated with insulin resistance, i.e. there is suppressed or delayed responses to insulin in insulin sensitive tissues. Hormones, cytokines and metabolic fuels from the adipocyte can diminish insulin action. Large adipocytes in obese subjects are resistant to insulin suppression of lipolysis, particularly in visceral fat. This all results in elevated levels of fatty acids and glycerol, which exacerbate insulin resistance in skeletal muscle and liver [38].

1.3 GENETICS OF COMPLEX DISEASES

Many human diseases have a genetic component. When searching for genetic variants that predispose to common disease the strategy depends on the underlying genetic model, which is often unknown [39]. Polygenic diseases often cluster in families and are strongly inherited but do not show simple heredity patterns. It is likely that both common and rare variants are contributing to common disease [40-42]. Human genetic variation occurs as single nucleotide polymorphisms (SNPs) and specific combinations of alleles are known as haplotypes. SNPs are single changes of base pairs. They cover about 90% of the human sequence variation and are considered as major determinants of predisposition to complex diseases. Besides these sequence variabilities other epigenetic events (histone acetylation, RNA interference and DNA methylation) add to the different complex gene regulation in individuals [43].

1.3.1 Type 2 diabetes

Polygenic or multi-factorial T2D is a result of the interaction between many genes and the environment. The susceptibility to T2D is associated with frequent polymorphisms that influence expression of genes in regulatory parts and create amino acid changes in proteins [44-45]. Such alleles of genetic variants are present in healthy subjects and T2D patients with different frequencies and are associated with a limited increase in the risk of developing disease. The SNPs are considered as susceptibility variants but they are not causative factors. Polygenic T2D is normally

diagnosed in the later stages of life [46]. The complex nature of T2D makes it harder to identify individual genes associated with increased risk of diabetes. To date, genetic studies have together identified 27 confirmed and potential common variants associated with T2D [47]. Eight of these loci appear to be involved in β-cell insulin secretion and response to an increased insulin resistance or obesity. One gene is involved in insulin sensitivity, another in glucose transport and two are related to obesity. The rest have unknown roles [48]. A major T2D susceptibility gene, transcription factor 7-like 2 (*TCF7L2*), has been identified and shown to account for 20% of T2D cases [49]. The gene is associated with alterations in insulin secretion. It has also been found that common variants in the *TCF7L2* gene can be used together with major histocompatibility complex, class II, DQ beta 1 (*HLA-DQB1*) genotyping to distinguish between young adults with antibody-positive and antibody-negative diabetes. *HLA-DQB1* is a gene that is associated with an increased risk of developing T1D. This is not possible in middle-aged diabetic patients, suggesting that middle-aged antibody-positive patients are different from young antibody-positive patients and that T1D in middle-aged patients shares genetic features with type 2 diabetes [50].

1.3.2 Obesity

Obesity is a heterogeneous disease and many common genetic variants contribute to the risk of developing this disorder, each conferring a modest odds ratio. The obesity risk is greatly affected by functional genetic variations and environmental factors, as in the case with T2D. Gene-environment interactions means that the combined effect of genotype and environment results from the additive or multiplicative effects of both factors [43]. Environmental factors are modifiable whereas genetic factors are not [51]. In the majority of patients with obesity, multiple genes interact with many environmental factors over time. As the technology advances the list of susceptibility genes to obesity grows. The influence of genes that have been associated with obesity is modest [52]. The strongest associated SNP in obesity is rs9939609 in the fat mass and obesity associated gene (*FTO)*. This variant has about 1% effect on the variance seen with BMI [53].

1.3.3 Study approaches

There are three basic strategies for disclosing and characterizing genes that influence complex diseases: genome-wide linkage scan, candidate gene analysis and genome-wide association studies. Each of these strategies has unique advantages, features, motivations and problems associated with them and will therefore be discussed separately.

1.3.3.1 Genome-wide linkage scan

Genome-wide linkage scan is a method used to search for possible genes that are responsible for diseases in human. It is family-based and investigates if any genetic markers (microsatellites or SNPs) from a set of markers that spans the whole genome co-segregate with disease phenotypes. It is based on finding a statistical signal that gives the probability of co-segregation of a disease with a chromosomal locus. If a signal is present it is said to be linked to the trait investigated. When a chromosomal region has been identified the next step is to search within this region for recombination events in one or several families [54-55]. The search for mutations is performed in all genes located in the particular region despite their biological role. This step is known as positional cloning. Sequence mutations are checked for their role in disease and this is done by verifying if they co-segregate with disease in linked families. One also looks at the mutations in the control group and tries to define their role in disease by biological

experiments [56]. Supports for disease association are segregation with disease in families, lack of mutation in controls or alteration of protein function. One example of successful gene identification by this approach is the MODY3 story [56-57]. It has not been as fruitful in finding T2D loci, with the exception of calpain 10 [58-59] and TCF7L2 [49]. More than 60 genome scans have been performed for obesity and 250 quantitative trait loci (QTLs) were identified by 2006 [60]. A meta-analysis of 37 published studies including more than 31,000 subjects did not detect strong evidence for BMI or BMI-defined obesity at any locus [61]. Linkage analysis is only successful for common diseases when variants have strong effect in one gene. Since replication of loci from common disease has been tough, it further suggests that these variants only have small genetic effects.

1.3.3.2 Candidate gene association

This approach examines specific genes with a potential role in the disease pathophysiology. Candidate genes are selected based on their role in biological pathways relevant for a phenotype of interest. It has the advantage of a short-range effect compared to linkage and it can be carried out in unrelated individuals, which are normally easier to recruit than families. The candidate gene approach has broadly been used to study the genetic basis of pharmacogenomic traits. Most success has been found in cancer where positive impact on patient care is seen [62]. Genetic association studies are much more powerful than linkage studies in finding common variants of modest effect [63]. Many candidate genes are derived from animal models, which are extremely useful since there is limited availability of human tissue. Today there are animal models of insulin resistance, obesity, β-cell dysfunction, impaired glucose homeostasis, insulin secretion and T2D. They provide important insights into human disease and are making great progress in identifying genes that may be functionally essential in the pathophysiology of obesity and T2D [64]. Two T2D genes that have been detected by candidate-gene association are the peroxisome proliferator-activated receptor gamma (*PPARG*) gene [65] and the potassium inwardly rectifying channel, subfamily J, member 11 (*KCNJ11*) [66]. Both genes are involved in targets of T2D drugs.

1.3.3.3 Genome-wide association study

Genome-wide association study (GWAS) is a hypothesis-free method of investigating the association between common genetic variation and disease. This type of study requires a dense set of markers (i.e. SNPs) that cover a great proportion of common variants across the genome and large number of subjects [35]. GWAS are very similar to genome-wide linkage as far as being a hypothesis-generating approach. It involves screening of the whole genome and the aim is to identify new genetic variants associated with disease. GWAS uses a case-control design to increase the chances of recruiting large numbers of subjects and also to gain statistical power. The statistical analyses identify top hits for SNPs and candidate genes which are then targeted for additional genotyping in a larger independent cohort of cases and controls for replication [35].

Recently, there have been many advances that have made GWAS successful. One of them is the sequencing of the human genome [67] and the International HapMap project [68-69]. It has increased our knowledge of common genetic variation and linkage disequilibrium (LD). The other is the development of high-throughput genotyping. Today it is possible to genotype millions of SNPs. The SNP chips available for this technique capture more than 80% of the common genetic variations reported in

HapMap [70]. The first GWAS of diabetes was published in 2007 [71] and soon after that several groups simultaneously published GWAS [72-75].

There are limitations of GWAS. It is based on the common disease-common variant hypothesis, which means that common diseases are caused by a few common variants instead of many rare variants. This theory has recently been discussed [76]. It is of interest to note that the majority of the results from GWAS are for markers that are not in known genes [35]. Identification of cases and controls for GWAS also has limitations. If young subjects are used their metabolic function can be normal at the time of the study but risk allele carriers may develop age-related phenotypes later in life [77]. Several loci that confer predisposition to T2D have recently been discovered and replicated by a number of genome-wide association studies [71-75]. In general, the GWA approach has increased the number of obesity- and T2D-associated markers substantially. Many of the associations found by GWAS have poorly understood functions. Now it is important to identify causal variants and distinguish their biological role in disease.

1.4 IMPACT OF CANDIDATE GENES ON OBESITY AND TYPE 2 DIABETES

This thesis is focused on the candidate gene approach. It includes genetic association studies of obesity and T2D. Candidate genes are derived from previous studies by our group. They are also selected based on their role in biological pathways relevant for pathogenesis of obesity and T2D. The candidate genes studied are described in the same order as in the list of publications.

1.4.1 Receptor protein tyrosine phosphatase σ

Receptor protein tyrosine phosphatase sigma (RPTPσ) is a member of the leukocyte antigen-related (LAR) RPTP family, which has been suggested to act in key steps of neural development and also in diabetes and cancer [78-79]. LAR, a protein closely related to RPTPσ, has negative regulatory effects in the insulin signaling pathway when it is over-expressed [80]. The *RPTPσ* gene gives rise to several different distinct isoforms [81]. PTPs are key regulators of the insulin receptor signal transduction pathway and the *RPTPσ* gene has been shown to be highly expressed in insulin target tissues, such as liver, adipose tissue, skeletal muscle, and endothelial cells [82]. In humans, the *RPTPσ* gene is located on chromosome 19p13.3, a region that may influence traits underlying lipid abnormalities associated with T2D [83].

We have previously demonstrated that the *RPTPσ* gene is over-expressed in pancreatic islets and liver of spontaneously diabetic Goto–Kakizaki (GK) rats that is an animal model of T2D mainly characterized by impaired insulin secretion. The GK rat is non-obese and develops mild hyperglycemia early in life. Its glucose intolerance is most likely due to impaired β-cell function together with polygenic inheritance [84]. When islet *RPTPσ* was inhibited by antisense, improved glucose-induced insulin secretion was seen in GK islets [85]. In addition, *RPTPσ* knockout (-/-) mice has shown decreased plasma glucose and insulin levels in the fasted state when compared with wild-type controls. The mice also had increased whole-body insulin sensitivity, suggesting that RPTPσ affects insulin signaling in insulin-sensitive tissues [86]. Until now, there has not been a genetic report of *RPTPσ* in T2D patients.

1.4.2 Zinc-α_2-glycoprotein 1

Zn-α_2-glycoprotein 1 (ZAG/AZGP1) is a 43-kDa soluble protein that recently has been classified as a novel adipokine and appears to play many important roles in the human body. *AZGP1* expression is regulated by TNF-α and the PPARγ receptor [87-89]. The gene is 9.3 kb long, includes 4 exons and is located on chromosome 7q22.1 [90-91]. AZGP1 is homologous to lipid mobilizing factor (LMF), which shares similar chemical identity and biological activity with AZGP1. Both proteins have been associated with loss of adipose tissue stores in cancer cachexia and shown to stimulate lipolysis by adipocytes both *in vitro* and *in vivo* [92-93]. AZGP1 is thought to be involved in regulation of body weight and genetically affected obesity [94]. It has the ability to induce uncoupling protein (UCP) expression in brown adipose tissue and skeletal muscle [95]. This process is most likely related to the loss of adipose tissue. Gohda et al. reported *AZGP1* to be a strong candidate gene for obesity using genetically homogenous T2D KK/Ta mice [96]. The KK/Ta strain is a polygenic mouse model for the common form of T2D associated with obesity in humans. Another study has demonstrated that *AZGP1* deficient mice are overweight and their adipocytes appear to have decreased lipolysis [97]. Treatment with *AZGP1* stimulates lipolysis in both human and mouse adipocytes and reduces body fat in normal and ob/ob mice [98]. Recently, studies have shown decreased serum levels of AZGP1 in obese subjects[99-100].

1.4.3 Adrenergic receptor α 2A

The sympathetic nervous system (SNS) plays an important role in regulating metabolism of glucose and lipids [101]. In addition to direct effects on metabolic substrate fluxes, SNS modulates release of insulin and glucagon which in turn regulate metabolism of glucose, lipids and protein. Catecholamines increase glucose levels by stimulating glycolysis and by decreasing peripheral glucose uptake as well as by inhibiting insulin release and stimulating glucagon secretion. Catecholamines are the most important regulators of lipolysis in human adipose tissue [102-103]. Thus, stimulation of β-adrenergic receptors enhances lipolysis whereas stimulation of α_2-adrenergic receptors inhibits lipolysis [102-103]. Insulin inhibits catecholamine-stimulated lipolysis by reducing the effects of adrenaline on β_2- and activating α_2-adrenergic receptors in adipocytes [104]. Low activity of the SNS is a risk factor for weight gain and obesity [105]. The sympathetic tone later increases in obese individuals and this will have adverse effects on pancreatic function and may contribute to the abnormal glucose-induced insulin secretion in obese subjects[106]. Increased activity of SNS also contributes to development of hypertension and elevated cardiovascular risk in obese subjects [107].

As described above catecholamines exert an important physiological role by α_2-adrenergic receptor mediated inhibition of insulin secretion in animals and in man. In contrast, stimulation of β_2-adrenergic receptors enhances insulin release [108-110]. Increased expression of α_2-adrenergic receptors in β-cells can cause alterations in insulin secretion regulation and contribute to etiology of T2D [111]. α_{2A}-adrenergic receptor deficient mice exhibit increased plasma insulin levels, reduced blood glucose levels and improved glucose tolerance [112].

Polymorphisms in the human α_{2A}-adrenergic receptor gene have been identified and associated with obesity [113-116], elevated glucose levels [117], reduced insulin secretion and increased risk of T2D [118], hypertension [119], cardiovascular diseases [120] and attention-deficit hyperactivity disorder (ADHD) [121]. However, the genetic susceptibility with obesity has only been investigated in a limited number of individuals.

1.4.4 Adenylate cyclase 3

Adenylate cyclases (ACs) are enzymes that catalyze the synthesis of cyclic-adenosine mono-phosphate (cAMP) from adenosine tri-phosphate (ATP). At least nine closely related isoforms of ACs have been cloned and characterized in mammals [122]. AC3 is expressed in pancreatic islets, brain, heart, kidney, liver, lung and skeletal muscle. The protein consists of two transmembrane regions, each containing six predicted membrane spanning helices, and two cytoplasmic regions [122]. It is located on chromosome 2p23.3 [123].

The GK rat exhibits a markedly reduced glucose-induced insulin release *in vivo* and in isolated perfused pancreas and isolated islets [124-127]. A previous study from our laboratory demonstrated that *AC3* mRNA was over-expressed in pancreatic islets of the GK rat, which was caused by two point mutations at in the promoter region [128]. The insulinotropic effect of forskolin in GK rat islets is associated with an enhanced cAMP generation and with over-expression of *AC3* mRNA [129]. Moreover, liver AC activity was increased in the membranes of male ob/ob mice in comparison to lean control mice [130]. These findings suggest a role for the AC3 gene in the pathogenesis of T2D and obesity. There is no reported study of genetic association with the AC3 gene in T2D, obesity or metabolic syndrome.

2 AIMS

The overall aim of this thesis was to find genetic association of SNPs in the selected candidate genes and to better understand their role in the development of obesity and T2D.

Paper I - RPTPσ

- To evaluate the genetic influence of *RPTPσ* polymorphisms in development of T2D.

Paper II - AZGP1

- To investigate *AZGP1* differential expression in subcutaneous (S.C.) abdominal, omental and thigh adipose tissue to establish the role of AZGP1 in obesity.
- To study *AZGP1* polymorphisms in association with obesity and/or T2D.

Paper III - ADRA2A

- To establish the role of *ADRA2A* genetic variation in T2D and/or obesity.

Paper IV- AC3

- To evaluate the association of *AC3* genetic variation in T2D and/or obesity.

3 SUBJECTS AND METHODS

3.1 SUBJECTS

3.1.1 Stockholm diabetes prevention program

Stockholm diabetes prevention program (SDPP) is a study which includes participants from four (in men) and five (in women) municipalities in the Stockholm region (Sigtuna, Tyresö, Upplands-Bro, Värmdö (Upplands-Väsby)). The participants were aged 35-56 years when entering the study. The study consists of a baseline study in men 1992-1994 and in women 1996-1998. A follow-up study in about 70% of the baseline participants was conducted 8-10 years later [6-7 131-132]. The subjects underwent a standardized 75 g oral glucose tolerance test (OGTT) after an overnight fast. Venous blood samples were taken before and 2 hours after glucose ingestion. Abnormal glucose regulation was diagnosed according to the World Health Organization criteria (WHO,1998) [133]. Current standard definitions of overweight and obesity were used according to the Center for Disease Control (CDC, 1998) and World Health Organization (1997) [134-135]. All individuals were unrelated. Among the included subjects 50% had a positive family history of diabetes (FHD) where FHD was defined as having at least one first-degree relative (parent or sibling) or at least two second-degree relatives (grandparents, aunts or uncles). All clinical data in NGT controls (with no FHD), IGT obese and T2D subjects were used from the baseline study (for those who were obese/T2D at baseline and those who became obese/T2D between baseline and follow-up studies i.e. incident cases) or from the follow-up study (for those who were diagnosed obese/T2D at follow-up). Baseline data for incident cases were used to avoid the influence of lifestyle changes and/or anti-diabetic treatment on phenotypes. Informed consent was obtained from all participants before initiation of the study. The procedures followed were in accordance with the declaration of Helsinki II and approved by the ethics committee of Karolinska Hospital.

3.1.2 Subjects for microarray analysis

A total of 21 men (46-63 years) with NGT were studied. The obese subjects (n=11) had a BMI > 30 kg/m^2. The lean subjects (n=10) had a BMI of 20-23 kg/m^2 and no FHD. They were matched regarding age to the obese group. All individuals were Swedish Caucasians collected from SDPP [131-132 136]. After an overnight fast, adipose tissue biopsies of 3-4 g were taken surgically from the subcutaneous (S.C.) abdominal region with local anesthesia and then frozen until gene expression analysis. A whole blood sample was taken from the subjects and was used for DNA extraction and variation screening. All participants gave their informed consent and the ethics committee of Karolinska Institutet approved the experimental protocol.

BMI was calculated as weight (in kg) divided by the square of height (in m^2). Body composition was measured by dual energy X-ray absorptiometry (DEXA) using a total body scanner (DPX-L; Lunar Radiation, Madison, WI, USA) and maximal oxygen uptake (VO_2max) using a bicycle ergometer exercise test. Whole body insulin sensitivity was measured using the euglycemic hyperinsulinemic clamp technique [137-138]. A 2h 75 g OGTT was performed after a 12 h overnight fast. Diabetes was defined according to the criteria of the WHO 1998 [133]. Anthropometric and metabolic characteristics for each subject group are given in Table 1. As expected the lean and obese groups differed with regard to BMI, WHR, % total fat and % truncal fat but not

for age and VO_2 max. The obese had lower insulin sensitivity than their non-obese counterparts.

Table 1. Data on subjects used for microarray gene expression profiling

Clinical parameters	Lean (n=10)	Obese (n=11)	*P-value*
Age	54.6±6.3	56.6±2.7	0.397
BMI (kg/m^2)	22.2±1.3	33.1±2.2	<0.001
Waist-hip ratio	0.87±0.0	1.0±0.1	<0.001
Fasting plasma glucose (mmol/l)	4.7±0.3	5.0±0.3	0.073
2 h plasma glucose (mmol/l)	4.5±0.5	5.4±0.9	0.014
M-value clamp	8.7±1.8	4.2±1.3	<0.001
VO_2 max	41.0±6.2	37.6±6.4	0.338
Total fat (%)	15.0±3.7	28.9±3.0	<0.001
Truncal fat (%)	44.0±7.8	52.8±2.9	0.012

All data are means ± SD. P-value was calculated by a Student's t-test.

3.1.3 Subjects for gene expression by real-time PCR

Samples used for microarray analysis were not available for real-time PCR due to limited amounts of RNA. Instead a total of 10 obese and non-obese subjects (41-82 years) were studied during cholecystectomy. The obese subjects (n=6, 2 men/4 women) had a BMI range of 31.1-34.2 kg/m^2. The lean subjects (n=4, 1 man/3 women) had a BMI range of 20.7-25.0 kg/m^2 and no family history of diabetes. They were matched regarding age to the case group. All individuals were Swedish Caucasians collected from Ersta Hospital, Stockholm, Sweden. After an overnight fast, adipose tissue biopsies of 3-4 g were taken surgically from the S.C. abdominal and thigh regions as well as omental (visceral) fat and then frozen until gene expression analysis. All participants gave their informed consent and the procedures followed were in accordance with the declaration of Helsinki II and approved by the ethics committee of Karolinska Hospital.

3.2 METHODS

3.2.1 DNA extraction

DNA was extracted from peripheral blood using Puregene DNA purification kit (Gentra Systems, Minneapolis, MN, USA). The Gentra Systems Puregene DNA purification kit is used for purifying genomic, mitochondrial, and viral DNA. It includes alcohol and salt precipitation. The first step is to lyse cells with an anionic detergent in the presence of a DNA stabilizer that inhibits DNase activity. After that RNA and proteins are digested and removed together with other contaminants by salt precipitation. The DNA is then precipitated with alcohol and dissolved in a DNA stabilizer.

3.2.2 SNP selection and validation

We have selected SNPs from the National Center for Biotechnology Information (NCBI) (USA; http://www.ncbi.nlm.nih.gov/SNP/) database based on validation status, region, and function. SNPs chosen were picked to cover the whole genes. The Tagger program from the International HapMap project was also used to select and evaluate tagSNPs from genotype data in HapMap [139]. Here, pair wise tagging was used together with an r^2 cutoff of 0.8 and a minor allele frequency (MAF) of 5%. The majority of the chosen SNPs were captured by tagger. SNPs were also chosen based on previously studied populations and results obtained during variation screening (papers II and IV). To validate the selected SNP genotyping assays in our population we used 32 Swedish

DNA samples including 16 obese subjects and 16 NGT controls. SNPs representing at least 4-5% allele frequencies were used for further genotyping in the larger sample set.

3.2.3 Genotyping

3.2.3.1 Dynamic allele-specific hybridization

High throughput genotyping was performed by dynamic allele-specific hybridization (DASH) (MCA System, ThermoHybaid, UK) [140]. The DASH principle consists of dynamic heating and monitoring of DNA denaturation. No additional enzymes or reaction steps are involved. The DASH-assay is carried out in a micro-titer plate format and uses fluorescence signal detection. The target sequence is amplified by PCR. One primer is labeled with biotin. The biotinylated product strand is bound to a streptavidin-coated micro titer plate well, and the non-biotinylated strand is rinsed away with NaOH. An oligonucleotide probe, specific for one of the alleles, is hybridized to the target at low temperature. This forms a duplex DNA region that interacts with a double strand-specific intercalating dye. Upon excitation, the dye emits fluorescence proportional to the amount of double-stranded DNA (probe-target duplex) present. The sample is then steadily heated while fluorescence is continually monitored. A rapid fall in fluorescence indicates the melting temperature of the probe-target duplex. A single-base mismatch between the probe and the target results in a dramatic lowering of melting temperature (T_m), which is detected. PCR-DASH assay design and SNP genotyping protocol were used as described previously [141].

3.2.3.2 TaqMan allelic discrimination

TaqMan Allelic Discrimination is a probe technology that uses the 5´-3´ nuclease activity [142] of AmpliTaq Gold ® DNA Polymerase to allow detection of the PCR product by releasing a fluorescent reporter. AmpErase ® UNG is required for the prevention of PCR product carryover [143]. The allelic discrimination assay requires two probes, one for each allele. Each probe consists of an oligonucleotide with a 5´-reporter dye and a 3´-quencher dye. TET (tetrachlorofluorescein) is covalently linked to the 5´ end of the probe for the detection of allele 1. FAM (carboxyfluorescein) is covalently linked to the 5´-end of the probe for the detection of allele 2.

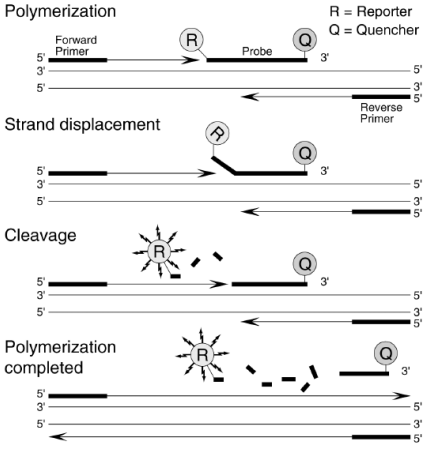

Figure 2. The mechanism of TaqMan Allelic Discrimination. The 5´–3´ nuclease activity of AmpliTaq Gold DNA polymerase during the extension phase of PCR is shown [144-145]. Picture from TaqMan allelic discrimination protocol 4303267D.

The reporters are quenched by TAMRA (tetramethylrhodamine), which is attached at the 3´-end of each probe. When the probe is intact, the close distance of the reporter dye and quencher dye results in suppression of reporter fluorescence. Forward and reverse primers hybridize during PCR to a specific sequence of the target DNA. The TaqMan probe hybridizes to a target sequence within the PCR product. The AmpliTaq Gold polymerase cleaves the TaqMan probe. The reporter- and quencher-dye are separated when the probe is cleaved ad this results in increased fluorescence from the reporter, see Figure 2. The 3´ end of the TaqMan probe is blocked to prevent extension of the probe during PCR. This event occurs in every PCR cycle and does not interfere with the exponential increase of product. The increase in fluorescence is measured, and is a direct consequence of target amplification during PCR. Both primer and probe must bind to their targets for amplification and cleavage to occur. The fluorescence signals are created only if the target sequences for the probes are amplified during PCR [146-147]. Therefore, non-specific amplification will not be detected. High throughput genotyping was performed by TaqMan allelic discrimination (ABI 7300, Applied Biosystems, USA)[148-149]. TaqMan assay design and SNP genotyping protocols were used as described previously [141 150].

3.2.3.3 Pyrosequencing

To confirm our genotyping data in paper IV, particular for those SNPs that were not in Hardy–Weinberg equilibrium (HWE), we have used another high-throughput genotyping method, that is pyrosequencing (PSQ96t, Pyrosequencing AB, Sweden) using the same primers as in DASH. Pyrosequencing is a sequencing method based on real-time monitoring of DNA synthesis. It is a four-enzyme DNA sequencing technology detected by bioluminescence [151]. In the first step, a primer is hybridized to a single-stranded PCR product and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase as well as the substrates, adenosine 5' phosphosulfate (APS), and luciferin. In step 2, the first deoxyribonucleotide triphosphate (dNTP) is added to the reaction. If the dNTP is complementary to the base in the template strand it is incorporated. The incorporation is catalyzed by DNA polymerase. Every incorporation is followed by release of pyrophosphate (PPi) in a quantity reflecting the amount of incorporated nucleotide. In step 3, ATP sulfurylase converts PPi to ATP in the presence of adenosine 5' phosphosulfate (APS). The ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in proportion to the amount of ATP. The light is detected by a charge coupled device (CCD) chip and seen as a peak in the raw data output (Pyrogram). The height of each peak is proportional to the number of nucleotides incorporated. In step 4, apyrase, a nucleotide-degrading enzyme, degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added. In step 5, addition of dNTPs is performed sequentially. It should be mentioned that deoxyadenosine alfa-thio triphosphate (dATP·S) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram.

SNP determination starts with analysis of nucleotide(s) preceding the investigated position. The advantage of using cyclic addition of nucleotides is that it results in three distinctive patterns at the polymorphic sites due to non-synchronized extensions. In contrast, the sequential nucleotide addition generates differences in three peak positions and is designed so that the individual allele extensions are in

phase. Thereafter, further nucleotide additions will give the consensus sequence of the target and can improve raw data interpretation [152].

3.2.4 Microarray gene expression profiling

The microarray technique has made it possible for global measurement of gene expression on mRNA level (transcript level). This experiment uses oligonucleotide arrays, which are developed by Affymetrix, Inc (Santa Clara, CA, USA). The output from the arrays can be used in many analysis programs, where genes can be selected, filtered and clustered for further examination. Microarray is a specific, sensitive and reproductive technique. The human genome U95_Av2 gene chips contain probe sets interrogating approximately 12 600 gene transcripts [153]. Microarray expression profiling consists of the following steps [154]:

- Target preparation – dsDNA is synthesized from total RNA, isolated from tissue. *In vitro* transcription (IVT) is then performed to generate biotin-labeled cRNA from cDNA.
- Target hybridization – A hybridization cocktail is prepared and it includes probe array controls, fragmented target, bovine serum albumin (BSA) and herring sperm DNA.
- Probe array washing and staining.
- Probe array scan – After washing and staining the array is scanned. Each probe array is scanned twice and the software combines the two images. It also defines the probe cells and gives the intensity for each cell. This double scan has the advantage that it improves assay sensitivity and it also reduces background noise.
- Data analysis – Initial data is analyzed using Microarray Suite Expression Analysis. The data is then transferred to another program for further analysis.

Total RNA from 500-1000 mg S.C. abdominal adipose tissue was isolated using the TriZol reagent (Life Technologies, Gaithersburg, MD) and the Fast RNA green (BIO101, Vista, CA) kit according to the manufacturer's protocols. The RNA samples were purified using the RNeasy kit (Qiagen, Valencia, CA), quantified with a spectrophotometer. Absorbance at 260 nm and 280 nm were used for determination of sample concentration and purity. Samples were then analyzed by agarose gel electrophoresis for 18S and 28S rRNA to verify integrity of the RNA. RNA was visualized by including ethidium bromide in the gel. For microarray analysis labeled cRNA was synthesized from total RNA according to the standard Affymetrix protocol (Affymetrix, Santa Clara, CA) and hybridized to human genome U95_Av2 gene chips. For one lean subject, RNA material was not sufficient to be used for hybridization. Possible outliers were identified by principal component analysis (PCA) [155]. Among the 20 microarrays hybridized, 16 passed quality control. Nine microarrays from obese subjects and seven microarrays from lean subjects were used for further statistical analysis. The raw files containing the fluorescence probe intensity information were summarized into gene signals using the RMA algorithm [156]. Genes differentially expressed between obese and lean subjects were identified using a two-tailed Student's t-test. Due to the large number of tests performed, a false discovery rate (FDR) based multiple testing procedure was applied to control the rate of false positives [157]. A FDR of 5% were chosen to declare a significant difference.

3.2.5 RNA extraction and real-time PCR

Human fat tissue was collected and frozen at -80 °C. Tissues were disrupted using a Mini Beadbeater (Biospec Products, Bartlesville, OK) with 500 µl 2 mm Zirconia beads and further processed with the RNeasy Mini kit protocol according to the manufacturer (Qiagen, Valencia, CA). The RNA integrity was checked by running a 1.5% agarose gel and the RNA concentration was determined by measuring the absorbance at 260 nm in a spectrophotometer. RNA quality was assessed measuring the A_{260}/A_{280} ratio. cDNA transcription was performed using the Quantitect Reverse Transcription kit (Qiagen, Valencia, CA). Gene-specific primers and probe (Assay ID Hs00426651_m1) for real-time PCR were obtained from the Applied Biosystems assay on demand service and used according to manufacturer's protocols [158]. Real-time PCR was performed in an ABI 7300 system (Applied Biosystems). 18S was chosen as a reference gene for normalization. Target amplification was performed using the following program: 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min for 40 cycles. Experiments were replicated once i.e. two measures per sample. Gene expression data were analyzed using the relative quantification method followed by the standard curve method. Differences in expression levels between study groups are described as fold-change/difference to the reference gene. A t-test was performed to determine expression differences between study groups.

3.2.6 Variation screening

3.2.6.1 Paper II

Variation screening was performed in a total of 188 subjects. For 167 subjects, DNA samples were obtained from the Coriell Institute for Medical Research (Caucasian panel and a panel of diabetic and non-diabetic subjects with family history of diabetes). Twenty one subjects were Swedish men from which both S.C. abdominal fat (for microarray profiling) and whole blood was used. Genomic DNA from these subjects were extracted from whole blood using MagNA Pure LC DNA Isolation Kit 1 (Roche Applied Science, Basel, Switzerland) and fragments targeting the complete sequence of *AZGP1* gene were amplified using 384 Cleanup Filter plates (Millipore, Billerica, MA). Variation screening and analysis was performed in the 188 subjects using sequencing analysis based on Big Dye terminator chemistry (Applied Biosystems, ABI model 3730, Foster City, USA). After sequencing, variation detection was performed using Polyphred software (University of Washington, Washington, DC). A total of 29 variants in the *AZGP1* gene were identified and 15 of them had a minor allele frequency of ≥ 5%. Some SNPs were completely redundant and therefore not taken into account.

3.2.6.2 Paper IV

Genomic DNA was extracted from peripheral blood by using a Puregene DNA purification kit (Gentra, USA). Screening for variation in the putative promoter region of the AC3 gene was performed with a protocol of direct sequencing analysis. A set of DNA samples extracted from 40 T2D patients and 8 non-diabetic control subjects in Swedish Caucasians were used. The PCR products were purified using MicroSpin HR columns (Amersham Biosciences, Piscataway, NJ, USA) and examined using a Big-dye sequence kit (Applied Biosystem, ABI model 377 genetic analyzer, Perkin-Elmer, Foster City, USA).

3.2.7 Calculations and statistical analysis

3.2.7.1 Hardy-Weinberg equilibrium

The HWE principle states that both allele and genotype frequencies in a population remain constant, which mean that they are in equilibrium. This occurs from generation to generation if not disturbing influences are introduced. Disturbing influences are non-random mating, mutations, selection, limited population size, random genetic drift and gene flow. HWE is impossible in nature. Genetic equilibrium is an ideal state that provides a baseline to measure genetic change against. Testing deviation from HWE was performed by a χ^2-test, using the observed genotype frequencies obtained from the association data and the expected genotype frequencies obtained using HWE.

3.2.7.2 Homeostasis model of assessment (HOMA)

The homeostatis model of assessment (HOMA) is a method used to quantify insulin resistance and β-cell function. It was first described under the name HOMA in 1985 [159]. In this thesis the HOMA was used to assess insulin resistance. Based on fasting glucose and insulin levels, it was calculated according to the following equation: fasting plasma glucose (mmol/l) x fasting plasma insulin (mU/ml)/22.5 [159].

3.2.7.3 Data preparation

Normal probability plots were created and parameter distributions were transformed to natural logarithm as required to improve skewness and to obtain a normal distribution before performing statistical analysis. Homogeneity of variances was tested by Levene's test, which is an inferential statistical method used to assess the equality of variance in different samples. Some common statistical procedures assume that variances of the populations from which different samples are drawn are equal. Levene's test assesses this assumption. It tests the null hypothesis that the population variances are equal. If the resulting p-value of Levene's test is less than some critical value (typically 0.05), the obtained differences in sample variances are unlikely to have occurred based on random sampling. Thus, the null hypothesis of equal variances is rejected and it is concluded that there is a difference between the variances in the population.

3.2.7.4 Subgroup analysis

For comprehensive analysis (papers I, II and IV), T2D patients were divided into subgroups based upon a BMI cutoff of 30 kg/m^2, that is non-obese T2D patients with BMI <3 0 kg/m^2 and obese T2D patients with BMI ≥ 30 kg/m^2. Since most of T2D patients are obese or overweight, a limited number of T2D patients had BMI ≤ 26 kg/m^2.

3.2.7.5 Allelic association

Genetic association between groups and different SNPs was performed using an Armitage's trend test [160] or χ^2-test. Armitage's trend test is often used as a genotype-based test for case-control association studies. OR and 95% confidence intervals (CI) were calculated to test for relative risk for association.

3.2.7.6 Analysis of variance (ANOVA)

ANOVA gives a statistical test of whether the means of several groups are all equal, and therefore generalizes Student's two-sample *t*-test to more than two groups. Test for differences in clinical parameters between genotype groups was performed by using ANOVA and covariance analysis adjusting for age. P-values less than 0.05 were interpreted as statistically significant.

3.2.7.7 Multiple logistic regression

Multiple logistic regression is used for prediction of the probability of occurrence of an event by fitting data to a logistic curve. It is a generalized linear model used for binomial regression. It makes use of several predictor variables that may be either numerical or categorical. Logistic regression is used extensively in the medical sciences. Multiple logistic regression analysis was used to predict the susceptibility to T2D between case and control subjects. Adjustments for age, sex, BMI, and blood pressure (where appropriate) were applied.

3.2.7.8 Linkage disequilibrium and haplotype analysis

LD is an allelic association where risk alleles of two different SNPs/markers that are close to each other co-segregate in one haplotype with frequencies different than expected. LD is the non-random association of alleles at two or more loci, not necessarily on the same chromosome. LD describes a situation in which some combinations of alleles or genetic markers occur more or less frequently in a population than would be expected from a random formation of haplotypes from alleles based on their frequencies. Non-random associations between polymorphisms at different loci are measured by the degree of LD.

The effects of the studied SNPs were tested individually and/or as haplotypes. LD and haplotype frequencies were calculated using Arlequin [161] or the Haplotype program (EH-plus) (ftp://linkage.rockefeller.edu/software/eh). LD between different markers/SNPs was summarized using |D´|. Haplotypes prevalent at 5% were used for further haplotype analysis. In paper IV, haplotypes were analyzed as diplotypes. A diplotype is a pair of haplotypes.

All statistical analyses were performed using Statistica version 8.0 (Statsoft Inc., Tulsa, OK, USA) and/or Biomedical package (BMDP) version 1.1 (BMDP Statistical Software Inc., Los Angeles, CA, USA) and/or Partek software version 6.4 (Partek, St Louis, MO) or Statistical Analysis System (SAS), version 9.1 (SAS Institute, Cary, NC, USA).

4 RESULTS

4.1 TYPE 2 DIABETES

4.1.1 RPTPσ

This genetic association study evaluated the influence of polymorphisms of the *RPTPσ* gene in development of T2D among Swedish men and women. The cohort included 497 unrelated subjects with NGT (246 men/251 women), 262 subjects with IGT (107/155), and 298 patients with T2D (241/57). A total of 12 valid SNPs were genotyped by DASH and all of them were in HWE (P>0.05). Three SNPs were associated with disease, rs1143699, rs4807015, and rs1978237, see Table 2.

Table 2. Allelic association of SNP rs1143699 and rs1978237 in NGT, IGT subjects and T2D patients

SNP ID	Sex	Allele frequencies (%)			*P*-value	OR (95% CI)
		NGT	IGT	T2D	T2D	T2D
rs1143699	M	C84/T16	C86/T14	C89/T11	0.029	1.57 (1.05-2.36)
	F	C89/T11	C89/T11	C89/T11	NS	-
	M+F	C87/T13	C88/T12	C89/T11	NS	-
rs4807015	M	C51/T49	C49/T51	C47/T53	NS	-
	F	C59/T41	C55/T45	C53/T47	NS	-
	M+F	C56/T44	C52/T48	C49/T51	0.025	1.32 (1.03-1.68)
rs1978237	M	G71/C29	G65/C35	G79/C31	NS	-
	F	G73/C27	G74/C26	G78/C22	NS	-
	M+F	G72/C28	G70/C30	G80/C20	0.002	1.59 (1.18-2.13)

P-value represents allele frequency difference between NGT vs. T2D. NS: not significant, M: male, F: female.

SNP rs1143699 showed significant allelic association with male T2D patients when compared with NGT controls. Multivariate logistic regression analysis of SNP rs1143699 with adjustments for age, BMI, and blood pressure indicated that in men the C/C genotype was significantly associated with T2D when compared with C/T + T/T.

A significant difference in allele frequency was found in SNP rs4807015 comparing male and female T2D patients with NGT controls (0.49 vs. 0.56), C being the major allele present at a lower frequency in T2D. Multiple logistic regression analysis showed that this SNP was associated with increased risk of T2D in men and women carrying the C/C genotype adjusted for age and blood pressure.

SNP rs1978237 showed significant allelic association with male and female T2D patients when compared with NGT controls (0.80 vs. 0.72), where the major allele G was present at a higher frequency in T2D. Multiple logistic regression analysis showed that this SNP was associated with increased risk of T2D in men and women carrying the G/G genotype adjusted for age, BMI, and blood pressure.

4.2 OBESITY

4.2.1 AZGP1

Through microarray gene expression we detected a significantly decreased expression of *AZGP1* in S.C. abdominal fat in obese human subjects compared to lean subjects, Figure 3.

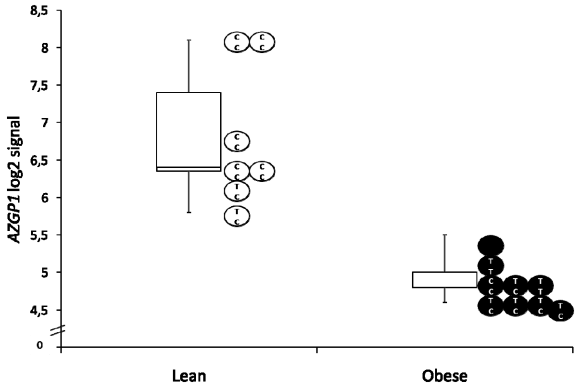

Figure 3. Box plot of *AZGP1* expression signals and rs2525554 genotypes in lean and obese subjects. Expression signal values are normalized and log2-transformed. Lean and obese subjects appear in white and black circles respectively. The corresponding genotype for each subject is displayed within the circles. For one obese subject genotype data was not available. P=3.32 x 10^{-5}.

We further investigated the differential expression of *AZGP1* in human abdominal, omental and thigh adipose tissue to establish the role of *AZGP1* in different regions of fat. As shown in Figure 4, *AZGP1* showed significantly decreased expression ratio in abdominal fat of obese subjects compared to lean. Thus, the differential expression between the two groups is 4.7-fold. *AZGP1* also showed significant down-regulation by 2.5-fold in omental fat in obese patients, but its expression did not differ between study groups in thigh fat, Figure 4.

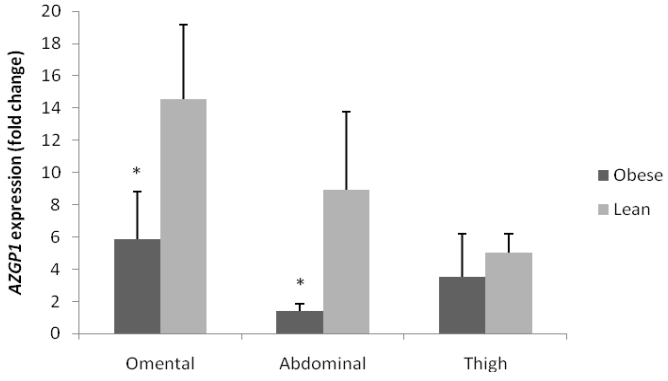

Figure 4. *AZGP1* gene expression levels in abdominal, omental and thigh fat. Gene expression data of *AZGP1* in obese (dark grey bars, n=6) and lean (light grey bars, n=4) subjects measured with real-time PCR. The levels in S.C. abdominal fat were down-regulated by 4.7-fold in obese compared to lean subjects, 1.9 vs. 8.9 (fold-change) respectively. In omental fat the levels were down-regulated by 2.5-fold in obese compared to lean subjects, 5.9 vs. 14.6 (fold-change) respectively. In S.C. thigh fat the expression levels did not differ between the two study groups, 3.5 vs. 5.0 (fold-change), P=0.32. *$P\leq$0.01. Error bars represent SD.

We also studied polymorphisms in *AZGP1* in association with obesity and/or T2D in 816 Swedish men, including 290 unrelated lean subjects with NGT, 199 obese subjects with NGT, 86 obese subjects with IGT and 241 subjects with T2D. A total of four valid SNPs in the *AZGP1* gene were genotyped by DASH and TaqMan. All of them were in HWE (P>0.05). SNP rs2525554 was associated with obesity in Swedish men.

Quantitative trait analysis revealed a significant association with increased BMI, waist circumference (WC), waist-hip ratio (WHR) and 2h glucose. Decreased *AZGP1* gene expression in obese subjects was related to their genotype for SNP rs2525554 as the risk allele T was seen more frequently among the obese compared to lean subjects, Figure 3.

4.2.2 ADRA2A

This study addressed the role of *ADRA2A* genetic variation in human T2D and/or obesity in a Swedish cohort including healthy controls with NGT and a BMI of ≤ 26 kg/m^2 (n=580; 394 men/186 women), IGT subjects with BMI between 21.8-44.8 kg/m^2 (n=109; 87 men/22 women), T2D patients with BMI between 18.4-46.2 kg/m^2 (n=399; 235 men/164 women) and obese subjects with NGT and BMI ≥ 30 kg/m^2 (n=198 men). We genotyped three SNPs in the *ADRA2A* gene by TaqMan allelic discrimination in our clinical material. Genotype distributions of all studied SNPs followed HWE. Test for associations in all SNPs were performed. We found associations linked to two of the studied SNPs, rs553668 in men and rs521674 in women.

SNP rs553668 in the *ADRA2A* gene showed significant association with obesity. The association was also seen when performing multiple logistic regression analysis adjusting for age. For a summary of association and multiple logistic regression of rs553668 in men, see Table 3.

Table 3. SNP rs553668: Genotype distribution and association in men

rs553668									
Comparisons	**Genotype distribution**			**Armitage's trend test**			**Multiple logistic regression G/G vs. A/A**		
	G/G	G/A	A/A	OR	CI 95%	*P*	OR	95% CI	*P*
Lean NGT vs. T2D	295/158	89/65	7/10	1.50	1.08-2.01	0.017	3.02	1.00-9.26	0.050[a]
Lean NGT vs. T2D							2.29	0.35-15.06	0.389[b]
Lean NGT vs. Obese NGT	215/134	67/55	5/9	1.51	1.03-2.07	0.034	2.91	1.00-8.87	0.061[a]
Lean NGT vs. Lean T2D	295/40	89/11	7/2	1.09	0.61-1.95	0.783	1.77	0.24-12.79	0.573[a]
Lean NGT vs. Obese T2D	295/118	89/54	7/8	1.61	1.14-2.22	0.007	3.80	0.92-2.62	0.056[a]
Obese NGT vs. Obese T2D	134/57	55/26	8/4	1.11	0.70-1.74	0.675	0.77	0.20-3.05	0.712[a]
Lean+Obese NGT vs. Lean+Obese T2D	427/157	141/65	14/10	1.33	1.00-1.76	0.061	1.96	0.76-5.09	0.165[a]

Adjusted by a) age and b) age and BMI

As to SNP rs521674 we found association between lean NGT subjects and obese T2D patients. Multiple logistic regression analysis was carried out and confirmed the association adjusting for age. We did not have access to obese women with NGT and could therefore not compare this group with lean women with NGT. We interpret the findings as indicating the association between SNP rs521674 and obesity in women but we were not able to exclude a link to T2D. Table 4 demonstrates tests for association and multiple logistic regressions for SNP rs521674 in women.

Table 4. SNP rs521674: Genotype distribution and association in women

rs521674									
Comparisons	**Genotype distribution**			**Armitage's trend test**			**Multiple logistic regression A/A vs. T/T**		
	A/A	A/T	T/T	OR	CI 95%	*P*	OR	95% CI	*P*
Lean NGT vs. T2D	106/103	60/54	14/3	4.54	1.27-16.25	0.012	7.43	1.66-33.26	0.008[a]
Lean NGT vs. T2D							2.59	0.39-16.93	0.322[b]
Lean NGT vs. Lean T2D	106/22	60/12	14/2	1.13	0.62-2.07	0.699	2.19	0.35-13.45	0.399[a]
Lean NGT vs. Obese T2D	106/81	60/42	14/1	1.94	1.00-2.25	0.050	9.42	1.02-87.33	0.048[a]
Lean+Obese NGT vs. Lean+Obese T2D	106/102	60/54	14/3	1.61	1.00-2.01	0.082	7.38	1.64-33.14	0.009[a]

Adjusted by a) age b) age and BMI.

4.2.3 AC3

In this study we investigated the association of T2D with *AC3* genetic variation in Swedish men. We carried out variation screening for the putative promoter region of human *AC3* gene and identified a novel SNP, -17A/T. A total of 630 unrelated men were included in this study. They were healthy control subjects with NGT and BMI ≤ 26 kg/m^2 (n=188), T2D patients with BMI 18.4-45.6 kg/m^2 (n=243) and obese subjects with NGT and BMI ≥ 30 kg/m^2 (n=199). We genotyped 14 valid SNPs, including the novel polymorphism, by DASH and TaqMan allelic discrimination. All studied SNPs were in HWE. The identified SNP –17A/T in the promoter region of the human *AC3* gene was not associated with disease. We found that two other SNPs, rs2033655 and rs1968482, had significant differences of allele frequencies when comparing control subjects and all T2D patients. Analyses for genotype differences showed that both SNPs, rs2033655 and rs1968482, were associated with obese T2D. We further genotyped the obese subjects with NGT and found that both SNPs rs2033655 and rs1968482 were strongly associated with obesity. Logistic regression analyses indicated a significant association with genotype distribution of these two SNPs in the obese subjects. Similar results were obtained when age was included in the model. Results described above are summarized in Table 5A and B.

Table 5. Association of SNPs rs2033655 (A) and rs1968482 (B) with T2D patients and obese subjects in comparison with control subjects

A. SNP rs2033655

Groups	**Genotypes**			**MAF (%)**	**Allelic association**			**Recessive model[c]**		
	T/T	T/C	C/C		OR	95% CI	*P-value*	OR	95% CI	*P-value*
Controls	34	96	51	C 0.55						
All T2D	31	101	98	C 0.65	1.509	1.14-2.00	0.004	1.892	1.28-2.87	0.003
								1.837	1.13-2.98	0.014[d]
T2D (BMI ≤26 kg/m^2)	11	22	17	C 0.56	0.817					0.425/0.842[d]
T2D (BMI <30 kg/m^2)	21	65	56	C 0.62	1.370	1.00-1.88	0.051	1.660	1.04-2.65	0.033/0.127[d]
T2D (BMI ≥30 kg/m^2)	10	36	42	C 0.68	1.775	1.22-2.59	0.003/0.202[a]	2.327	1.37-3.95	0.002
								2.360	1.26-4.41	0.007[d]
Obese	30	89	80	C 0.63	1.384	1.04-1.85	0.028/0.195[b]	1.714	1.11-2.64	0.014
								1.744	1.13-2.69	0.012[d]

B. SNP rs1968482

Groups	Genotypes			MAF (%)	Allelic association			Recessive model[c]		
	A/A	A/G	G/G		OR	95% CI	*P-value*	OR	95% CI	*P-value*
Controls	65	91	28	A 0.60						
All T2D	107	103	20	A 0.69	1.475	1.11-1.97	0.008	1.593	1.07-2.37	0.022/0.078[d]
T2D (BMI ≤26 kg/m^2)	20	28	4	A 0.65	0.325					0.678/0.924[d]
T2D (BMI <30 kg/m^2)	60	68	13	A 0.67	0.084					0.185/0.435[d]
T2D (BMI ≥30 kg/m^2)	47	35	7	A 0.73	1.571	1.19-2.59	0.005/0.190[a]	2.049	1.23-3.43	0.006
								2.018	1.11-3.68	0.022[d]
Obese	101	77	20	A 0.71	1.586	1.18-2.14	0.003/0.622[b]	1.906	1.26-2.88	0.002
								1.913	1.27-2.89	0.002[d]

a) comparison analysis for T2D (BMI ≥ 30 kg/m^2) vs. T2D (BMI < 30 kg/m^2). b) comparison analysis for Obese vs. T2D (BMI ≥ 30 kg/m^2). c) without adjusting for age. d) age was included in the regression model. MAF=Major allele frequency.

Analyses of haplotypes and diplotypes were also performed. Four haplotypes were constructed from the two associated polymorphisms. Haplotype frequencies in controls were significantly different from T2D and obese subjects. Diplotype association analyses revealed a significant association with BMI in obese subjects, Figure 5. The result was the same after adjusting for age.

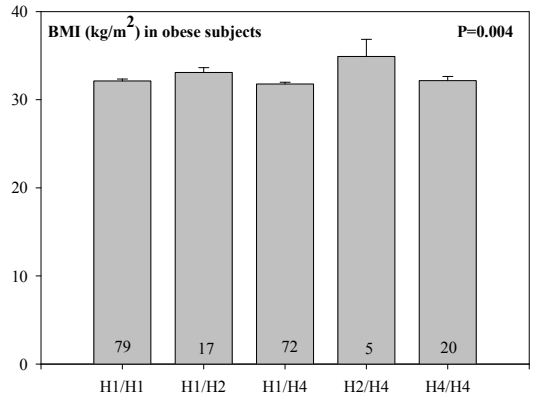

Figure 5. Diplotype association with BMI in obese subjects. Data were mean values (±SE) of BMI according to diplotype groups. The number of individuals in each haplotype is shown in the base of each column. ANOVA P-value is shown.

5 DISCUSSION

5.1 GENETIC EFFECTS OF AZGP1, ADRA2A AND AC3 IN OBESITY

5.1.1 AZGP1

AZGP1 is lipid mobilizing factor, which directly stimulate lipolysis *in vitro* through the cAMP pathway [162]. AZGP1 causes loss of body weight in two outbred strains of mice, NMRI and MF-1, generally used for physiology. This phenomenon was also seen previously for LMF [93]. *AZGP1* is also expressed by human adipocytes and regulated by TNF-α and PPARγ, suggesting a role in inflammation and insulin sensitivity. Furthermore, these two factors regulate adiponectin in mouse, suggesting a functional relationship between adiponectin and *AZGP1* [96]. In concert with our findings, recent studies have investigated the expression of *AZGP1* in S.C. abdominal and omental fat of obese and lean subjects and found that *AZGP1* was down-regulated in obese subjects [100 163-164]. We found that in lean subjects, *AZGP1* expression in S.C. thigh fat was lower than in the other locations, and with no different expression as compared with obese subjects, suggesting that S.C. thigh fat plays a subordinate metabolic role. It is possible that a decrease of both expression and activity of AZGP1 in white and brown adipose tissue accounts for reduced rates of lipolysis and UCP function [92-93 95]. *AZGP1* is thus involved in lipid metabolism and can be seen as a candidate gene in obesity regulation [89 99-100 163-164].

5.1.2 ADRA2A

SNP rs553668 has been reported to be associated with risk of obesity in Caucasians and African Americans [114-116], hypertension in African Americans [119] and with endurance in athletes of Caucasian origin [165]. This polymorphism has also been associated with childhood ADHD, hypertension and platelet aggregation [121 166-167]. Our study confirms the association with SNP rs553668 and obesity in Swedish men.

Recently, it was reported that SNP rs553668 is associated with reduced insulin secretion and increased risk of T2D [118] in a population from Finland and Southern Sweden. The association was verified on functional level in human pancreatic islets. Risk allele carriers showed over-expression of *ADRA2A* in islets, decreased insulin secretion and reduced number of docked insulin granules *in vitro*. The defects were corrected by ADRA2A-antagonism [118]. Taking into consideration these findings and our present study, we propose that *ADRA2A* is a common component in obesity and T2D, which may allow future specific therapy for individual patients and personalized medicine.

Variants in the β_3- and β_2-adenergic receptors have previously been associated with obesity and diabetes in several studies [168-171]. There is a major interest in the receptor variants and their association with altered function and etiology of disease. Many candidate genes have been proposed for obesity and yielded conflicting associations with obesity-related traits [60]. The reason for this might be sample size, different selected populations, gene-gene and gene-environment interactions or lack of reproducibility.

5.1.3 AC3

It has been demonstrated that AC/cAMP/protein kinase A axis is involved in regulation of both insulin release and lipolysis. Important incretin hormones such as glucagon-like

peptide (GLP-1), gastric inhibitory polypeptide (GIP) and β-adrenergic agonists are known to stimulate insulin release by increasing synthesis of cAMP [130 172-174]. In humans, the most important regulators of lipolysis are catecholamines, which stimulate mobilization of fat through β-adrenergic receptors and generation of cAMP, whereas α_2-adrenergic receptors suppress cAMP generation and lipolysis. cAMP influences may be important in regulating pancreatic islet β-cell function, differentiation, growth and survival [175]. ACs 1, 3 and 8, are most abundantly expressed in the brain and islets, where the cells need an elevated cAMP level to respond to multiple stimuli [103 176-177]. AC3 may play an interactive role with GLP-1, GIP and adrenergic receptors in control of insulin release and lipolysis. Therefore, a comprehensive analysis of AC3 in regulation of signaling by GLP-1, GIP and adrenergic receptors is necessary in order to understand the cellular mechanisms behind the postulated influence of *AC3* genetic variation on BMI and other related metabolic features in T2D and obesity.

Our results suggest that AC3, which is expressed in adipose tissue and the hypothalamus, may play an important role in the regulation of body weight. Another group has studied this hypothesis by disrupting the *AC3* gene and monitoring the weight of AC3-/- mice over an extended period of time to see if they develop obesity. The mice phenotype is consistent with the hypothesis that AC3 generates a cAMP signal in the primary cilia of the hypothalamus that is important for regulation of weight and leptin sensitivity [178]. The study by Wang et al. report that mice lacking AC3 exhibit obesity which is caused by low locomotor activity, hyperphagia and leptin resistance. The phenotype of the AC3-/- mouse is consistent with our data implicating a role of *AC3* polymorphisms in human obesity.

5.2 GENETIC INFLUENCE OF RPTPΣ IN TYPE 2 DIABETES

LAR is a member of the same PTP family as RPTPσ and has a role in negative modulation of insulin receptor signaling [179]. Increased activity of LAR or related PTPs in insulin target tissues could contribute to development of insulin resistance [180]. The fact that RPTPσ shows similarity to LAR, 85% at amino acid level, and is expressed to a higher level than LAR in insulin-sensitive tissues, makes this protein a possible candidate for regulating insulin signaling and for playing a role in glucose homeostasis [181]. To our knowledge, the present study is the first genetic investigation of RPTPσ in T2D. However, there is a genetic association study of an SNP in the promoter region of LAR in two non-diabetic populations in Italy, showing association with diabetes-related characteristics [182]. Thus, LAR and RPTPσ seem to be important genes in the development of T2D.

5.3 CANDIDATE GENE APPROACH

No results from candidate gene studies have reached genome-wide significance and meta-analysis did not improve the outcome [35]. Candidate gene association studies have not produced univocal results but the results that exist are strong enough if associations found are replicated, suggesting that many of these variants have a modest effect on obesity and T2D [35].

One difficulty with this approach is that many studies are small and therefore underpowered. The smaller the effect of a genetic variant and the lower the minor allele frequency, the larger sample size is required to identify an association with sufficient power.

The candidate gene approach will in the future continue to contribute to our understanding of obesity and T2D susceptibility, because it can perform more detailed analyses of biological relevant genes and their interaction with other genes and the environment. It requires large-scale cohorts and meta-analyses to confirm detected gene-disease associations. Associations should be looked at in populations with different environments and ethnic backgrounds. Negative association in one environment/ethnicity does not exclude positive association in another.

The individual effect of each genetic variant is often small and explains only a portion of the total heritability of the disease [183]. For several genes associated with complex diseases results have not been replicated and this raises concerns to design and interpretation of studies. Any association testing needs to include statistical power estimates, sample size and case-control stratification [184-190]. Careful study design can reduce study bias and false-positive results can be minimized. By controlling the probability of type I error there is also a possibility of ignoring important biological findings [191]. Replication should be carried out in a larger sample set from an independent study population with the same genetic background, phenotype and SNPs. Replication can be performed in a population of different genetic background to strengthen the evidence for true association. Although, if no association is seen it should not be taken as false positive evidence [192].

Even though GWA studies have brought a lot of new information, large-scale candidate gene studies will still be essential in the near future. The costs of SNP chips have decreased, although not everyone will be able to afford to genotype their samples by this technique. The candidate gene approach can evaluate specific genes in greater depth by the selection of tagSNPs or by sequencing a gene of interest. SNP chips do not capture all common genetic variation of the genome yet and they only cover a part of all rare variants. It might be rare rather than common variants, or a combination of both, that accounts for the variability of complex traits [193].

5.4 GENOME-WIDE ASSOCIATION STUDIES

After the initial GWAS and meta-analysis of most of them, there have been speculations about the clinical use and impact of the results. Many of the identified T2D loci seem to be related to β-cell function. These findings are partially related to the selection process of cases and controls in the GWAS. Two of the studies [71 75] selected lean cases to control for obesity. By minimizing the influence of obesity and therefore also insulin resistance, the chance of identifying insulin secretion genes increased. Another study that did not control for obesity found an association with *FTO*. When BMI was taken into account the association disappeared, which suggests that the association of *FTO* with T2D is mediated by obesity [53]. However, it has also been found that *FTO* variants are associated with insulin resistance in obese children and adolescents independent of BMI [194].

The GWA studies are today using markers with a minor allele frequency over 5%, which fails to detect rare variants of large effect [35]. It is important to remember that the contribution of genetic variants to obesity is estimated to be small. This could be due to the current study designs, which include specifically selected obese subjects where the main phenotype studied is BMI. There are other obesity-related phenotypes that could give important information. Candidate gene studies are able to focus on specific subgroup populations such as different ethnicities or children, investigate more detailed traits and study interactions with the environment.

The large data sets generated by GWAS are a challenge when it comes to analysis and interpretation. In the future GWAS also need to be extended to other populations to shed light to shared and population specific variants. The majority of the GWAS so far have been performed in North America or Western European individuals [35]. The majority of SNPs in commercially available arrays does not affect protein structure and seems unlikely to affect gene expression. This limits the discovery to finding chromosomal locations instead of genetic variants [195]. Fine mapping and sequencing is required for identifying functional variants [191 195-197].

We will not be able to elucidate the genetic architecture of common obesity and T2D by GWAS alone. We need alternative study designs and additional phenotype data to find new genes [35]. The GWA approach needs to be experienced further and some issues remains to be considered. Study design, number and selection of SNPs, population selection, statistical analyses are potential hurdles [184 198-200].

5.5 INTRONIC POLYMORPHISMS AND THEIR BIOLOGICAL RELEVANCE

All but one of the presently associated variants with T2D is located in non-coding regions. Whether these variants have effects on regulatory elements and gene expression is still unclear. Many of the identified genes are located near genes that could affect β-cell function. This can be a reflection of the study design of GWA studies, which focus on individuals with established T2D and thus β-cell dysfunction. It is of importance to study subjects with insulin resistance as the primary phenotype. Interestingly, many genes are widely expressed throughout the body. They could therefore have roles in tissues that are not investigated in clinical studies [77].

The majority of the associated variants in this thesis are located in introns. Two of the associated variants in the *RPTPσ* gene are located in intron 13 and 34, the associated variant in the *AZGP1* gene is located in intron 2 and the two associated variants in the *AC3* gene are located in introns 1 and 2. In other genes, intronic SNPs have been suggested to contribute to gene expression, and therefore, this is a possible explanation for the role of these SNPs as well [59 201-202]. Susceptibility is often a result of the combined action of many different variants in a gene, one example being calpain-10 in T2D [203]. Thus, the susceptibility of a single gene is small. It is the contribution of several genes combined with environmental components that will give rise to disease [188] . These considerations need to be emphasized when interpreting association data of genetic association studies.

One of the associated variants in the *ADRA2A* gene is located in the 3´-region. Genetic variations in the 3´-region can be important in regulation of message stability. Alteration in this region of the *ADRA2A* gene can affect gene expression by causing an increased stability of the mRNA [204]. The other associated SNP in the *ADRA2A* gene is situated in the 5´-region. Due to its location, this polymorphism has previously been described and used as a genetic marker [205-206]. Genetic variants found in the 5´-flanking region could be involved in transcription binding sites in the promoter region and influence gene expression. There is little information on *in vivo* effects of studied SNPs in the *ADRA2A* gene. The variants found are mainly non-coding and there is limited knowledge on their functional significance.

5.6 GENDER SPECIFIC ASSOCIATIONS

SNP rs1143699 in the *RPTPσ* gene was found to be associated with T2D in men but not in women, suggesting that gender differences may be an important factor in the genetic influence of *RPTPσ* in T2D. Our group and others have previously shown that there are sex differences in genetic association with T2D. Polymorphisms in the insulin-degrading enzyme (*IDE*) gene are associated with increased BMI and insulin resistance in men but not in women [207]. Also, a polymorphism in neuropeptide Y (*NPY*), a gene involved in satiety and body weight, is related to IGT and T2D in men [208]. Thus, sex specificity could be an important confounding factor in analyzing genetics of T2D and related disorders [136 207-212] and at least partly explain the higher prevalence of T2D in men vs. women in ages 35-65 years [6 136]. We also detected gender specific associations in the *ADRA2A* gene, but because of the limited size of our obese female samples in this study the results should be evaluated further and replicated in larger populations. Hence, gender seems to be an important factor to take into account when analyzing genetics of obesity and related disorders.

5.7 STOCKHOLM DIABETES PREVENTION PROGRAM

We did not detect any association between genotype groups and clinical phenotypes for the SNPs in the *RPTPσ*, *ADRA2A* or the *AC3* gene. This is most probably because our male patient group contains clinical data at baseline also for patients who were diagnosed with T2D during the 8-10 years between baseline and follow-up studies. This subgroup of T2D men did not have diabetes at baseline, which explains the low fasting plasma glucose in the diabetes group (6.6 mmol/l). However, we chose not to include follow-up data of these patients, since anti-diabetic drugs and lifestyle changes would then be affecting the quantitative traits of the patients. The same analysis approach was applied to all genetic association studies in this thesis.

5.8 GENE-GENE AND GENE-ENVIRONMENT INTERACTIONS

There is an increased awareness of the role of gene-gene interaction, or epistasis, in complex diseases. Epistasis is a common component of polygenic disorders and is thought to be more important than the independent effect of a susceptibility gene [213]. Physiological events are generally not regulated by a single gene. Linkage studies demonstrate chromosome 7q22.1- 7q35 to be a region of interest for association to obesity [214]. In addition to *AZGP1*, this region contains the leptin gene, encoding another body weight regulating hormone. Thus, it is of interest to study this gene region regarding interactions of *AZGP1* with leptin or other genes. Minor gene interactions might be essential for understanding obesity and T2D [94]. Studies have also found a positive correlation of *AZGP1* expression to serum adiponectin and a negative correlation with serum leptin, supporting previous findings that AZGP1 may affect the production of other adipokines [96 164].

It remains to be investigated how the *ADRA2A* association is regulated on a molecular basis in lipid and glucose metabolism. It is also of interest to study the effects of *ADRA2A* variants on response to administration of α_2-adrenoreceptor agonists and antagonists. Not only single but interactive effects of adrenergic receptor polymorphisms seem to contribute to obesity-related phenotypes such as hyperinsulinemia, insulin resistance and increased systolic blood pressure [215-216]. It is also important to study other components of the adrenergic receptor pathways in relation to the pathogenesis of T2D and obesity. The interest for gene-environment interactions is also growing. Non-genetic factors may play a major role in common

disorders. The prevalence of late-onset diseases have increased during the past decades and this increase is likely caused by non-genetic factors that trigger the disease in individuals that are genetically susceptible. Environmental factors have changed more recently than common disease alleles in the population [62]. Thus, other polymorphisms and environmental factors will give a better insight into the susceptibility, progression and treatment of obesity, T2D and related disorders.

5.9 GENETIC RESEARCH OF COMPLEX DISEASES

The advances in understanding the genome will be integrated into genetic studies. Once a genetic variant is identified further studies of epidemiological, genetic and physiological ground is needed. To determine which variants within a haplotype that are functionally related to disease, it is important to perform haplotype analyses on populations of different ethnicities by fine mapping the region of interest using sequencing in a large number of individuals. Expression studies will identify if the variants affect mRNA and protein levels. Since obesity and T2D are heterogeneous disorders, refining the phenotypic outcome can give more detailed information of the mechanisms that drive an association. It would be desirable to use more detailed additional phenotypic information such as diet, energy expenditure, physical activity, food intake, body fat percentage or fat distribution. This is essential if the genetic studies are going to provide information. Experiments at cellular level or in animal models will give us better understanding of molecular and physiological mechanisms and pathways that underlie an observed association. The genetic research of the last ten years have led to the identification of ~27 confirmed and potential T2D susceptibility genes [47].

Rare SNPs with a minor allele frequency of less than 5% have shown strong effects in common complex diseases such as obesity [217], T1D [218] and infection [219]. This suggests that both common and rare variants contribute to common disease. Copy number variants (CNVs) have been estimated to be causing 18% of the heritable variance in gene expression [220]. CNVs include copy number gains (duplications or insertions), losses (deletions) and rearrangements. Obesity associated SNPs in the neural growth regulator (*NEGR1)* gene have shown to be in strong LD with a nearby CNV [221].

A growing field is the regulatory role of microRNAs (miRNAs) [222]. They are small 22-bp non-coding RNAs that bind to target mRNA and modify their expression. One miRNA can regulate many targets and recently specific miRNAs have been found in the regulation of glucose and lipid metabolism [223-228]. It is of interest to study the contribution of genetic variance in miRNA coding and target sequences to T2D pathogenesis.

Epigenetics is the study of heritable changes in the genome that are not due to changes in the DNA sequence. The epigenetic changes are DNA methylation, histone methylation and chromatin modification. Imprinting is suggested to be linked to common polygenic obesity [229-230].

Other findings that seem to be very important to the concept of obesity and diabetes include the ”microbiome”, which includes hundreds of species of microbes. Germ-free mice suggest that the microbiome is important for the energy balance [231]. Furthermore, the brain has a major role in weight regulation and fat metabolism [232]. A new concept, named nutri-genomics, investigates food chemicals that may alter expression of genes that contribute to disease [233].

A lot more work is needed including larger-scale studies, studies using novel phenotypes, deep resequencing of the genome, studies looking at the role of CNV and epigenetics. The long-term goal is to identify causal variants and their biological function in obesity and T2D. Finally, the translation of all new genetic insights into clinical practice continues to be the overall goal and personalized treatment will be the next to conquer.

The genetic basis for obesity, T2D and other common diseases is complex. The susceptibility genes will be many and vary between families and ethnic groups. Will there be a common form of T2D that accounts for the majority of affected persons? The next 10 years will tell. When the important genes involved have been identified, the focus will be on developing therapies and to perform genetic testing on persons with disease and tailor the most effective treatment. Relatives can also undergo testing to determine their genetic predisposition and if they are at risk, treatment plans can be used for prevention [234]. It has been shown that patients with the *KCJN11* mutation respond better to sulfonylureas than insulin [235] and that carriers with the *TCF7L2* risk variant respond poorly to sulfonylureas [236]. Novel obesity and T2D genes will be potential pharmaceutical targets and the near future will identify more susceptibility genes for both disorders. Common traits are a large public health problem. The discovery of genetic profiles in individuals can be used to predict disease risk, prevention or treatment. This is one step towards personalized medicine.

6 CONCLUSIONS

Paper I – The study provides evidence that three polymorphisms in the *RPTPσ* gene are associated with the development of T2D in Swedish Caucasians, which suggests that the *RPTPσ* gene may be important for the development of T2D in humans.

Paper II – The study confirms that *AZGP1* expression is down-regulated in obese compared to lean subjects. We provide additional verification for the role of *AZGP1* in obesity by showing that the decreased expression is evident in S.C. abdominal and omental fat but not in S.C. thigh fat. SNP rs2525554 in the *AZGP1* gene is associated with abdominal obesity in Swedish men. Thus, *AZGP1* may play an important role in the pathogenesis of obesity.

Paper III – The study suggests that *ADRA2A* polymorphisms are associated with obesity and may also relate to T2D in Swedish Caucasians. Further investigation needs to be performed in other populations and functional effects of genetic variation should be analyzed.

Paper IV – The study provides the first evidence that *AC3* gene polymorphisms are associated with obesity in Swedish men with and without T2D. AC3 seems to play an important role in the regulation of body weight.

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